

**REMARKS**

Applicant's Attorney and Agent, Steven Davis and Michael Gottselig, would like to thank the Examiner and Supervisory Examiner for conducting the telephonic interview of July 2, 2007. Applicant refers to the Summary of Substance of Interview mailed July 30, 2007.

**Claim Amendments**

Claims 1, 2, 24, 25 and 26 have been amended as discussed with the Examiners (see above) to clarify that "transplanted" modifies both tissue and cell. Furthermore, the claims are amended to recite "subject in need thereof" to emphasize that the subject is in need of inhibition of rejection of a transplanted organ, transplanted tissue or transplanted cell.

Claims 27 and 28 have been added. Claims 27 and 28 differ from Claims 1 and 24, respectively, by the additional recitation of the inhibition of "acute rejection," that is, these claims are directed to a "method of inhibiting acute and chronic rejection of a transplanted organ, transplanted tissue or transplanted cell in a subject in need thereof." Support for these claims can be found, for example, in Claims 1 and 24 with, for example, the statement on page 3, lines 16 to 18.

**Claim Rejection under 35 U.S.C. § 112**

Claims 1-26 are rejected under 35 USC § 112 second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The Examiner asserts that the claims are indefinite because it is unclear whether the term "transplanted" applies to "organ" only or to "organ", "tissue" and "cell".

Applicants have amended Claims 1, 24 and 26 to recite "transplanted organ, transplanted tissue or transplanted cell" and Claims 2 and 25 to recite "transplanted organ or transplanted tissue." All other claims are dependent claims. Thus, Applicants respectfully submit that these amendments overcome the rejection of Claims 1-26 under 35 U.S.C. § 112 as discussed in the Interview.

Claim Rejection under 35 U.S.C § 102 (a)Claims 1-26

Claims 1-26 are rejected under 35 USC § 102(a) as being anticipated by Sneddon *et al.*, WO 01/87849. With respect to this rejection, Applicant respectfully notes that the Supervisory Examiner stated during the Examiner Interview that this rejection may not be valid and that the Examiner stated that she would reconsider Applicant's arguments provided in Applicant's previous response dated January 4, 2007 (see, e.g. Examiner's Interview Summary). Applicant refers to the arguments presented in Applicant's previous response filed January 4, 2007.

Applicant presented in his previous response three textbook references that teach that Graft Versus Host Disease (GVHD) and graft (or transplant) rejection are two entirely distinct and separate conditions. More specifically, 1) GVHD is caused by the graft reacting against the patient, 2) graft (or transplant) rejection is caused by cells of the patient, and 3) GVHD is not understood in the art to be encompassed by the term graft (or transplant) rejection and *vice versa*.

The Examiner states on page 2 of the instant Office Action:

In the interpretation that "transplanted" does not limit "tissue" or "cell", then GVHD rejection reaction in a patient would read on an embodiment of the instant claims.

Applicant respectfully disagrees with the Examiner, because the term "rejection" is not used in the art to refer to the action of a graft against its host (see previous response for more detail). Nevertheless, to expedite prosecution and for the sake of clarity, Applicant has amended Claims 1, 2, 24, 25, and 26 such that the term "transplanted" applies, independently, to "organ", "tissue" and "cell."

Thus, Claims 1 to 26, as currently amended, clearly do not encompass inhibition of GVHD.

The Examiner further states on page 3 of the instant Office Action:

It is the Examiner's view point that the risk of developing GVHD is attributed to a number of factors and that the risk increases with increasing amounts of lymphoid tissue being

transferred and the order of risk depends on the organ being transplanted as evidenced by Jamieson et al.

The Examiner does not explain how this view point and, in particular, the cited Jamieson et al. reference relates to the instant rejection of Claims 1-26 under 35 USC § 102(a) as being anticipated by Sneddon et al., WO 01/87849.

Applicant particularly emphasizes that Jamieson et al. is directed to GVHD in solid organ transplantation (see title) and teaches that one requirement of GVHD is the “inability of the host to reject the graft” (see page 67, left-hand side, first paragraph). Thus, a subject with GVHD, who is unable to reject the graft, is clearly not in need of inhibition of graft (or transplant) rejection. In contrast, the claims, as currently amended, are directed to either a method of inhibiting rejection of a transplanted organ, transplanted tissue or transplanted cell or to a method of inhibiting chronic rejection of a transplanted organ or transplanted tissue in a subject in need thereof. For this reason alone the claims, as currently amended, are novel in light of Sneddon et al.

#### Claims 2-23 and 25

Claims 2-23 and 25 are directed to a method of inhibiting chronic rejection of a transplanted organ or transplanted tissue in a subject in need thereof. Chalasani et al. (see Journal of Immunology, 2004, 172: 7813-7820; Exhibit D; page 7813, lines 1-14) teach with respect to acute and chronic rejection:

The host's immune response to donor Ags leads to **two types of allograft rejection that differ histologically and clinically. Acute rejection is characterized by an intense cellular and humoral attack on donor tissue that results in rapid graft loss. Chronic rejection in contrast is a more insidious process characterized by obliterative vasculopathy and parenchymal fibrosis that lead to progressive graft failure (1).** The risk of acute rejection in humans is highest in the early posttransplantation period, but declines dramatically over the ensuing months. **In contrast, the risk of chronic rejection increases gradually and**

**becomes a significant cause of graft loss after the first year of transplantation. (*emphasis added*)**

Accordingly, chronic rejection is 1) a type of transplant rejection, 2) differs histologically and clinically from acute rejection, and 3) becomes a significant cause of graft loss after the first year of transplantation.

Further, Hutchinson (Biomarkers and Surrogate Endpoints: Advancing Clinical Research and Applications, April 16, 1999; Natcher Conference Center, National Institutes of Health, Bethesda, Maryland; conference abstract available at <http://www4.od.nih.gov/biomarkers/b9.htm> ; see Exhibit E) teaches:

Acute cellular rejection is strongly associated with high-producer TNF-alpha genotype in heart and kidney recipients, whereas IL-10 and IFN-gamma play modulating roles. **Chronic rejection (including declining graft function, transplant vasculopathy, graft loss, and patient death) is strongly associated with high-producer TGF-beta 1 genotype. (*emphasis added*)**

Thus, TGF-beta 1 and not TNF- $\alpha$  is suggested to be the pivotal cytokine in chronic transplant rejection.

Sneddon *et al.* do not teach that the compounds described therein can be used to inhibit acute and chronic rejection of a transplanted organ or transplanted tissue in a patient in need thereof. Thus, for this reason alone, Claims 2-23 and 25 are novel in light of Sneddon *et al.*

In conclusion, for the reasons given above, independently, Claims 1-26 are novel in light of the Sneddon *et al.* reference. Accordingly, withdrawal of the rejection is respectfully requested.

#### Claim Rejection under 35 U.S.C § 103 (a)

##### Claims 1-26

Claims 1-26 are rejected under 35 USC § 103(a) as being unpatentable over Sneddon *et al.* (WO 01/87849) taken with Sviland *et al.* (J. Clin. Pathology 1999, 52:910-913) in view of Jamieson *et al.* (Transplant Int. 1991, 4:67-71).

Applicant respectfully disagrees with the Examiner's conclusion that the aforementioned prior art references render Claims 1-26 obvious.

None of the references cited by the Examiner teach or suggest administration of the compounds of the instant invention to inhibit rejection (and, in particular, chronic rejection) of a transplanted organ, transplanted tissue or transplanted cell in a subject in need thereof.

The Sviland et *al.* reference is directed to the prediction of GVHD following bone marrow transplantation based on a human skin explant model (see title). The Examiner explains the relevance of the Sviland et *al.* reference as follows (see page 6 of instant Office Action):

Sviland et al. teach GVHD is a complication following bone marrow transplantation (see abstract), wherein (tumor necrosis factor-alpha) TNF- $\alpha$  are important mediators of the cellular damage. (see abstract also)

Applicant respectfully submits that this teaching does not contribute to the teaching of Sneddon et *al.*, because it is already known from Sneddon et *al.* that GVHD is a TNF- $\alpha$  mediated condition. There is no teaching in Sviland et *al.* regarding transplant rejection or the compounds of the present invention.

The Examiner additionally explains the relevance of the Jamieson et *al.* reference as follows (see page 6 of instant Office Action):

Jamieson et al. teach that GVHD is a solid organ transplantation effect. The claims recite transplantation of an organ, tissue or cell (see abstract-highlighted sec.). This is well within the claim limitation.

It is again emphasized, that Graft Versus Host Disease (GVHD) and graft (or transplant) rejection are two entirely distinct and separate conditions (see detailed discussion in Applicant's previous response filed January 4, 2007.)

The Examiner further states that the motivation to combine Sneddon et *al.*, Sviland et *al.* and Jamieson et al. clearly comes from Jamieson et al. (see page 6 of instant Office Action). Again, Applicant respectfully disagrees. Although, all of the references include teaching

regarding GVHD, neither one nor a combination of these references provides motivation to combine them in the context of the inhibition of transplant rejection.

Furthermore, the teachings of Sneddon *et al.*, are limited to a method of treating a TNF- $\alpha$  mediated condition, for example, GVHD in a subject, and Jamieson *et al.* teach that a subject with GVHD is not in need of inhibition of transplant rejection. Thus, the combined teachings support the patentability and non-obviousness of the instant claims, because Jamieson *et al.* actually teaches away from using the compounds disclosed in Sneddon *et al.* to treat transplant rejection. Applicant notes that the Sviland *et al.* reference is irrelevant in this context.

#### Claims 2-23 and 25

Claims 2-23 and 25 are directed to a method of inhibiting chronic rejection of a transplanted organ or transplanted tissue in a subject in need thereof. As discussed above, Chalasani *et al.* teach that acute and chronic rejection exhibit divergent histological and clinical characteristics, for which the mechanisms are not well understood. Thus, independent of TNF- $\alpha$  being involved in the mechanisms of acute and chronic rejection or not, a person having ordinary skill in the art would not expect that the compounds taught by Sneddon *et al.* would treat both acute and chronic rejection. Again, Jamieson *et al.* and Sviland *et al.* do not contribute any teaching relevant in this context.

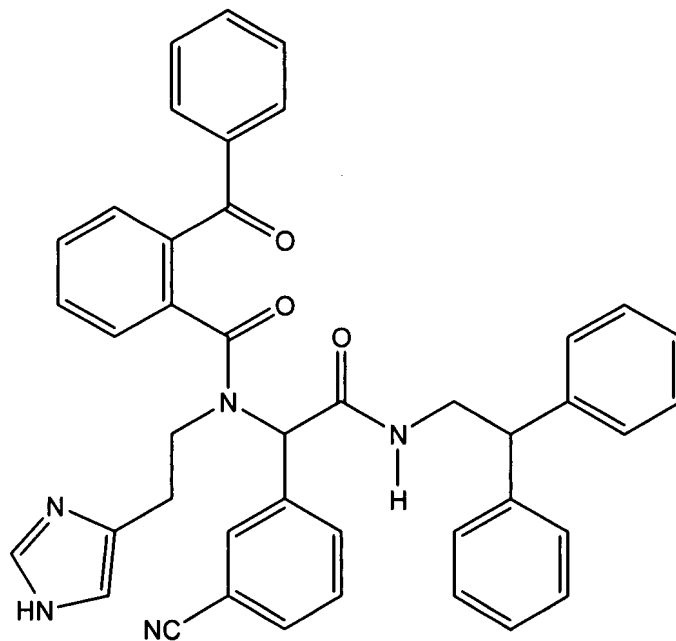
Furthermore and supporting the above, Goodman and Mohanakumar (Front. Biosci., 2003, Sept. 1;8:838-44; Exhibit F; see abstract) teach that although acute rejection rates have diminished markedly due to development of new generations of immunosuppressants, the challenge to prevent chronic rejection remains unsolved:

Current strategies for immunosuppression following organ transplantation focus on the prevention of acute rejection. **As new generations of immunosuppressants have been developed, acute rejection rates have diminished markedly. The new challenge, then, is to prevent the devastating complications of chronic rejection, which have remained largely unchanged over the decades.** The process of chronic rejection is a complex one, and it is likely that most, if not all, components of the immune

system play some role in the long-term, smoldering failure of organs following transplantation. (*emphasis added*)

The Applicant has now discovered that the compounds described in the instant specification are effective in preventing chronic transplant rejection. The instant specification page 2 lines 27 to page 3 line 12 states:

In one example, the histopathological evidence of chronic rejection was inhibited in two mouse models by Compound 1, shown below. In the first model, chronic rejection of fully MHC class II mismatched transplanted hearts in recipient mice at eight weeks post surgery was inhibited by treatment with 75 mg/kg/day of



Compound 1

Compound 1 alone during the two weeks following surgery. In the second model, chronic rejection of fully MHC class II mismatched transplanted hearts in recipient mice at 120 days post surgery was inhibited when treatment with 75 mg/kg/day of Compound 1 during the two weeks following surgery was combined with a single administration of 250  $\mu$ g of anti-CD154 monoclonal antibody immediately following transplant surgery. Treatment with anti-

CD154 monoclonal antibody alone suppresses acute rejection, but is ineffective in preventing chronic rejection of transplanted tissue.

In summation, while Sneddon *et al.*, have a generic teaching of a method of treating a TNF- $\alpha$  mediated condition in a subject there is no specific teaching of treating chronic transplant rejection. As discussed above, chronic transplant rejection is a specific type of transplant rejection, the underlying causes of which are not fully understood and for which there is currently no known treatment. The Applicant has discovered that the compounds described in the instant specification are effective in preventing chronic transplant rejection. Specifically, Applicant has found that the instant compounds inhibit chronic rejection of fully MHC class II mismatched transplanted hearts in recipient mice at eight weeks post surgery. Therefore, the instant invention represents a surprising and unexpected improvement in methods for inhibiting rejection of tissue transplants that could have not been predicted based on Sneddon *et al.* Again, Jamieson *et al.* and Sviland *et al.* do not contribute any teaching relevant in this context.

In conclusion, for the reasons given above, independently, the amended Claims 1-26 are non-obvious and patentable in light of Sneddon *et al.*, Sviland *et al.* and Jamieson *et al.*, and withdrawal of the rejection is respectfully requested.



**CONCLUSION**

In view of the above amendments and remarks, it is believed that all claims are in condition for allowance, and it is respectfully requested that the application be passed to issue. If the Examiner feels that a telephone conference would expedite prosecution of this case, the Examiner is invited to call the undersigned.

Respectfully submitted,

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# The Allograft Defines the Type of Rejection (Acute versus Chronic) in the Face of an Established Effector Immune Response<sup>1</sup>

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Transplanted organs fail due to either acute or chronic rejection. The prevailing view is that the nature or magnitude of the recipient's immune response to donor Ags determines the type of rejection. In variance with this view, we show in this study that the status of the graft itself plays a dominant role in defining the type of rejection even in the face of an established alloimmune response. Using adoptive transfer mouse models in which the graft is exposed to a constant number of effector lymphocytes, we found that newly transplanted heart allografts are rejected acutely, while healed-in allografts undergo chronic rejection. Acute rejection of healed-in allografts was largely recapitulated by subjecting the grafts to ischemia-reperfusion injury similar to that present in newly transplanted organs. Ischemia-Reperfusion injury altered the outcome of rejection by enhancing the accumulation of effector T cells within the graft. The accumulation of effector T cells in the graft was dependent on the presence of both ischemia-reperfusion injury (inflammation) and alloantigens. These findings demonstrate that the graft plays a dominant role in shaping the outcome of rejection by controlling the trafficking of effector T cells. *The Journal of Immunology*, 2004, 172: 7813–7820.

The host's immune response to donor Ags leads to two types of allograft rejection that differ histologically and clinically. Acute rejection is characterized by an intense cellular and humoral attack on donor tissue that results in rapid graft loss. Chronic rejection in contrast is a more insidious process characterized by obliterative vasculopathy and parenchymal fibrosis that lead to progressive graft failure (1). The risk of acute rejection in humans is highest in the early posttransplantation period, but declines dramatically over the ensuing months. In contrast, the risk of chronic rejection increases gradually and becomes a significant cause of graft loss after the first year of transplantation.

The mechanisms that underlie the divergent histological and clinical characteristics of acute and chronic rejection are not well understood. The prevailing view is that the nature or magnitude of the recipient's immune response to donor Ags determines the type of rejection that ensues. It is hypothesized that host exposure to intact MHC alloantigens displayed on donor APCs (direct allorecognition) results in acute rejection because of substantial expansion of T cells of multiple specificities, while host exposure to donor alloantigens processed and presented by host APCs (indirect allorecognition) leads to the activation of a limited T cell repertoire

with restricted ability to recognize graft targets, and thus, chronic instead of acute rejection (2–5). This hypothesis is supported by experiments in which blocking the direct allorecognition pathway or, alternatively, limiting the size of the alloreactive T cell clone shifted the rejection process from an acute to a chronic form (6–9). Furthermore, the abundance of donor APCs in newly transplanted allografts correlates with the high risk of acute rejection early after transplantation, while their gradual replacement with host APCs over time ushers in the period of chronic rejection (10, 11).

In addition to the transition from direct to indirect allorecognition during the afferent (sensitization) phase of the immune response, long-term surviving grafts undergo adaptive changes that protect them against the effector arm of the response (12). Graft adaptation was originally described by Woodruff and Woodruff (13), who found that thyroid allografts parked in the anterior eye chambers of guinea pigs are rejected if the recipients receive a simultaneous thyroid allograft under the skin, but become resistant to rejection if s.c. grafting is delayed by several weeks. Subsequent experiments provided evidence that skin allografts also become less vulnerable to rejection with time (14, 15). Despite these findings, the contribution of graft adaptation to the long-term survival of transplanted organs remains a matter of debate (16, 17), and its relative importance in defining the pattern of rejection (acute vs chronic) after the alloimmune response has been initiated is unclear. Using adoptive transfer models in which the graft is exposed to a constant number of effector lymphocytes, we demonstrate in this study that newly transplanted heart allografts are rejected acutely, while healed-in allografts survive long-term, but undergo chronic rejection. We also provide evidence that resolution of ischemia-reperfusion injury is a central mechanism of graft adaptation that protects vascularized organ transplants against acute rejection by limiting the accumulation of effector T cells within the graft.

## Materials and Methods

### Murine cardiac transplantation

All heart donors were 6- to 8-wk-old C3H (H-2<sup>b</sup>) or BALB/c (H-2<sup>d</sup>) mice, and all recipients were 6- to 8-wk-old C57BL/6 (H-2<sup>b</sup>) mice. All mice were

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purchased from The Jackson Laboratory (Bar Harbor, ME), except for alymphoplastic (*aly/aly*<sup>3</sup> and *aly/+* mice, which were purchased from Clea Japan (Tokyo, Japan). Heterotopic transplantation of primarily vascularized cardiac allografts was performed, as described (18). In this model, the pulmonary artery and ascending aorta of the heart graft are anastomosed to the recipient's inferior vena cava and abdominal aorta, respectively. Retransplantation of heart grafts was performed by anastomosis of donor (first recipient) aortic and inferior vena cuffs to the second recipient's aorta and inferior vena cava, respectively. The retransplantation procedure took ~80 min to complete with a total cold ischemia time of 20 min and warm ischemia time of 35 min. Mice were monitored daily. Rejection was defined as the cessation of palpable heart beat, at which time the graft was harvested for histological analysis. Wild-type allograft recipients received the following immunosuppression to prevent primary T cell activation: 0.25 mg of MR1 (anti-CD40 ligand mAb) and 0.25 mg of CTLA4-Ig (both generated in the laboratory of C. Larsen, Emory University, Atlanta, GA) i.p. on days 0, 2, 4, and 6 relative to heart transplantation.

#### *T cell preparation before adoptive transfer*

T cells were enriched from mouse lymph nodes and spleen by nonadherence to nylon wool (Polysciences, Warrington, PA) (19). Cell suspensions were subjected to hypotonic lysis of RBCs before passage on nylon wool. Final purity of the T cell population ranged between 75 and 85%. A total of  $4 \times 10^7$  enriched cells was transferred i.v. to each heart graft recipient at the indicated time points. To obtain activated lymphocytes, mice were immunized with  $2 \times 10^7$  allogeneic spleen cells i.p. and s.c. 4 days before harvesting the lymph nodes and spleen.

#### *Histological analysis*

Fixed, paraffin-embedded cardiac tissue was stained with H&E, Masson-Trichrome (MT), Verhoeff Van Gieson elastin stain, or anti-mouse CD3 (BD PharMingen, San Diego, CA), followed by peroxidase-conjugated secondary Ab. All analyses were performed by the pathologist (F.K.B.), who was blinded to the experimental protocol.

#### *Isolation of graft-infiltrating cells*

Heart allografts were perfused in situ with heparinized 0.9% saline, removed, minced, and digested in 20 ml of RPMI 1640 medium containing 10% FCS and 150 U/ml collagenase (Sigma-Aldrich, St. Louis, MO). The cell suspension was then passed down a loosely packed glass wool column to clear tissue debris, mixed with Percoll solution (Sigma-Aldrich) to a concentration of 35%, and centrifuged at 2000 rpm for 15 min at room temperature. The cell pellet was resuspended and washed in PBS and stained for flow analysis, according to standard procedures (20).

#### *Gene expression analysis by real-time PCR*

Total RNA was isolated and reverse transcribed, according to standard techniques (21). Direct detection of the PCR product was monitored by measuring an increase in fluorescence due to the binding of SYBR Green to dsDNA. Reactions were performed in a MicroAmp Optical 96-well reaction plate (Applied Biosystems, Foster City, CA) using for each separate well 5  $\mu$ l of cDNA mix, 5  $\mu$ l of primer, and 10  $\mu$ l of SYBR Green Master Mix (Applied Biosystems). Each well contained the primer pair for amplification of one of the parameters of interest. The gene-specific PCR products were continuously measured by means of the GeneAmp 5700 Sequence Detection System (Applied Biosystems) during 40 cycles. The threshold cycles, that is, the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected, of each target product were determined and set in relation to the amplification plot of GAPDH. All experiments were run in duplicate, and the same thermal cycling parameters were used. Nontemplate controls and dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. Fold change was calculated relative to control cycle threshold ( $C_T$ ). The  $C_T$  value is defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. With a PCR efficiency of 100%, the  $C_T$  values of two separate genes can be compared ( $DC_T$ ) and the fold difference =  $2 - (C_T - C_T^{\text{control}}) = 2 - DC_T$ .

#### *Gene expression analysis by oligonucleotide microarrays*

RNA isolation, cDNA synthesis, and cRNA transcription were performed, as previously described (22). cRNA was hybridized to Affymetrix murine

microarrays (Santa Clara, CA), which contain probe sets for 13,000 mouse genes. Hybridization, scanning, and data analysis were performed at the Affymetrix Gene Chip Core Facility in the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University (technical details are available at <http://info.med.yale.edu/wmkeck/affymetrix/>). Differentially expressed genes were identified by comparing BALB/c cardiac allografts harvested 2 or 50 days after transplantation into splenectomized *aly/aly* mice and before adoptive T cell transfer ( $n = 3/\text{group}$ ). Day 50 and day 2 allografts were also compared with native BALB/c hearts ( $n = 3$ ).

## Results

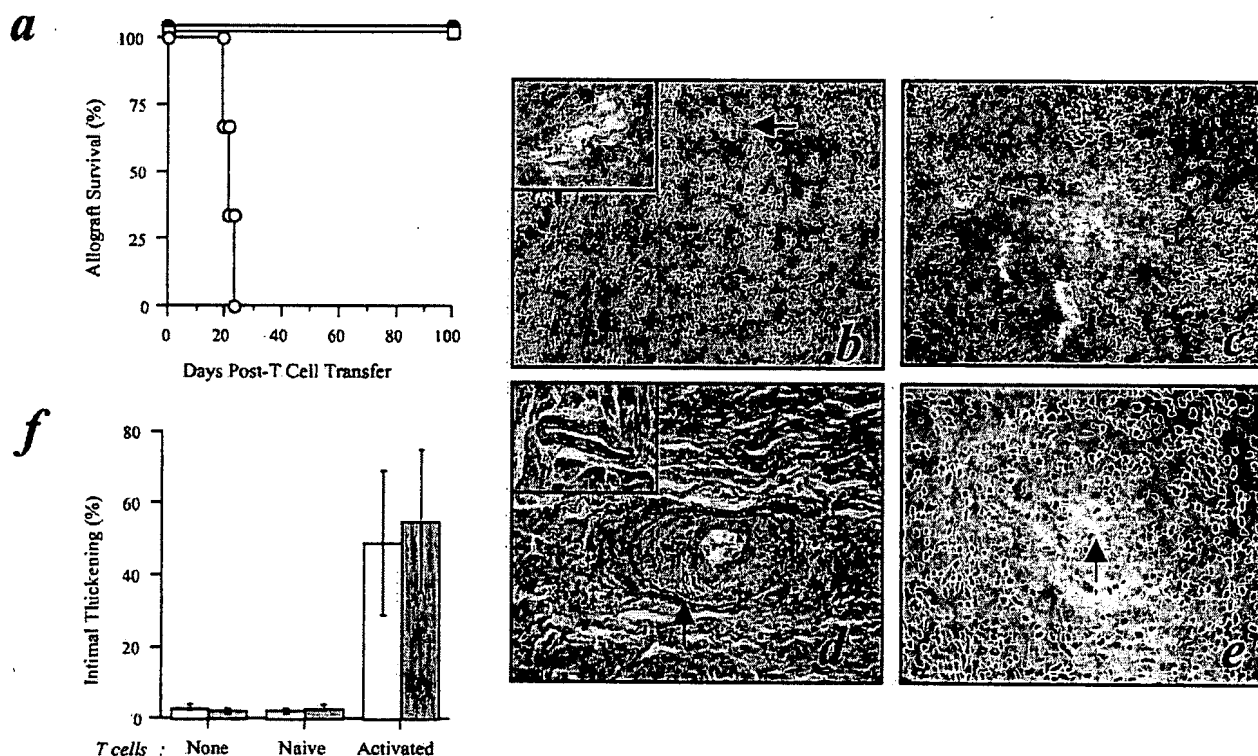
### *The status of the graft determines the type of rejection*

To investigate the extent to which the allograft influences the outcome of rejection, we used a heart transplantation model in which the status of the graft can be manipulated independent of the alloimmune response. In this model, we transplanted vascularized C3H (H-2<sup>b</sup>) hearts to splenectomized alymphoplastic mice (*aly/aly-spleen*; H-2<sup>b</sup>), which lack secondary lymphoid tissues and accept allografts indefinitely (23, 24). Exogenous activated T cells, obtained from B6 (H-2<sup>b</sup>) mice 4 days after immunization with C3H splenocytes, were then transferred to *aly/aly-spleen* recipients either 2 or 70 days after transplantation to precipitate allograft rejection (20, 24). Allografts were harvested on the day of clinical rejection (cessation of heart contractions) or 100 days after T cell transfer if the heart continued to contract. By varying the day on which activated T cells were transferred, this model allowed us to alter the status of the graft (newly transplanted vs healed-in), while keeping the afferent immune response (exogenous activated T cells) constant.

As shown in Fig. 1a, the transfer of activated T cells to *aly/aly-spleen* mice 2 days after transplantation invariably resulted in clinical rejection (all allografts stopped contracting within 23 days after cell transfer). In contrast, the transfer of an identical population of activated T cells 70 days after transplantation failed to precipitate clinical rejection in any of the *aly/aly-spleen* recipients (all allografts were still contracting 100 days after cell transfer). Rejection was not observed in control mice that received naive T cells either 2 or 70 days after transplantation (Fig. 1a). Histologic examination of cardiac allografts confirmed that the transfer of activated T cells 2 days posttransplantation resulted in high-grade acute cellular rejection. In contrast, delaying the transfer of activated T cells until day 70 posttransplantation led to a distinct form of allograft pathology characterized by the presence of diffuse interstitial fibrosis, obliterative vasculopathy, and perivascular T cell infiltrates (Fig. 1, b–e). Histometric analyses revealed that the obliterative vasculopathy was due to intimal thickening (Fig. 1f), a hallmark of the vascular lesion associated with chronic rejection (1). These data therefore indicate that, in the face of a constant effector immune response, a newly transplanted allograft undergoes acute rejection, while a healed-in allograft develops chronic rejection.

The results shown so far were generated in *aly* mice that harbor a mutation in the NF- $\kappa$ B-inducing kinase (25). In addition to the absence of secondary lymphoid tissues, this mutation is associated with immunologic abnormalities that could potentially influence allograft rejection mediated by exogenous effector T cells (26, 27). To rule out this possibility, we transferred activated T cells to wild-type recipients either 2 or 50 days after heart transplantation. The recipients were treated at the time of transplantation with CTLA4-Ig and MR1 to block the CD28/B7 and CD40/CD40 ligand costimulatory pathways, respectively. Because these pathways are required for the activation of naive, but not Ag-experienced T cells (28–30), this model allowed us to vary the status of the allograft (newly transplanted vs healed-in) in the absence of a

<sup>3</sup> Abbreviations used in this paper: *aly*, alymphoplastic;  $C_T$ , cycle threshold; Mig, monokine induced by IFN- $\gamma$ ; MT, Masson-Trichrome.



**FIGURE 1.** Healed-in allografts undergo chronic rejection in alymphoplasic hosts following the transfer of activated T cells. *a*, Cardiac allografts are acutely rejected in splenectomized *aly/aly* hosts if activated T cells are transferred 2 days after transplantation (○,  $n = 6$ ). In contrast, acute rejection is not observed if activated T cells are transferred 70 days after transplantation (□,  $n = 6$ ). The transfer of naive T cells on either day 2 or day 70 after transplantation does not lead to acute rejection (●,  $n = 4$ /group). *b–e*, H&E ( $\times 100$ ), MT ( $\times 200$ ), Verhoeff Van Gieson ( $\times 400$ ), and anti-CD3 ( $\times 400$ ) staining, respectively, of cardiac allograft tissue removed 100 days after T cell transfer in the mouse group that received activated T cells 70 days following transplantation. Note diffuse fibrosis (*b* and *c*), thickened vascular intima (*b* and *d*), and perivascular T cell cuffing (*e*), which are consistent with chronic rejection. Arrows demonstrate a vessel with thickened wall (*b*), internal elastic lamina and thickened vessel intima (*d*), and nearly obliterated lumen of a vessel (*e*) surrounded by T cells (brown stain). *Insets* in *b* and *d* depict normal allograft vessels from mice that received naive T cells 70 days after transplantation. *f*, Histometric measurements demonstrating that narrowing of vascular lumina in *aly/aly-spleen* mice that received activated T cells 70 days after transplantation is due to intimal thickening, a characteristic of chronic allograft vasculopathy (□, intima to wall ratio; ■, intima to lumen ratio). Data from control *aly/aly-spleen* mice that did not receive exogenous T cells or received naive T cells on day 70 are also shown. Allografts were harvested 100 days after T cell transfer.

significant host primary immune response. In addition, this model simulates the normal clinical setting in which graft adaptation and allograft rejection occur in immunosuppressed patients. As shown in Fig. 2*a*, mice that received activated T cells on day 2 rejected their allografts promptly, while those that received activated T cells on day 50 did not reject throughout the observation period (total of 100 days posttransplantation). As in the *aly* model, transferring activated T cells on day 2 led to acute rejection, while day 50 transfer caused chronic allograft rejection (Fig. 2, *b* and *c*). Control mice that were treated with CTLA4-Ig and MR1, but did not receive activated T cells, did not reject their allografts and had minimal vasculopathy on day 100 posttransplantation (Fig. 2, *d* and *e*). These results confirm, in a clinically relevant model, that graft adaptation plays a dominant role in defining the type of rejection.

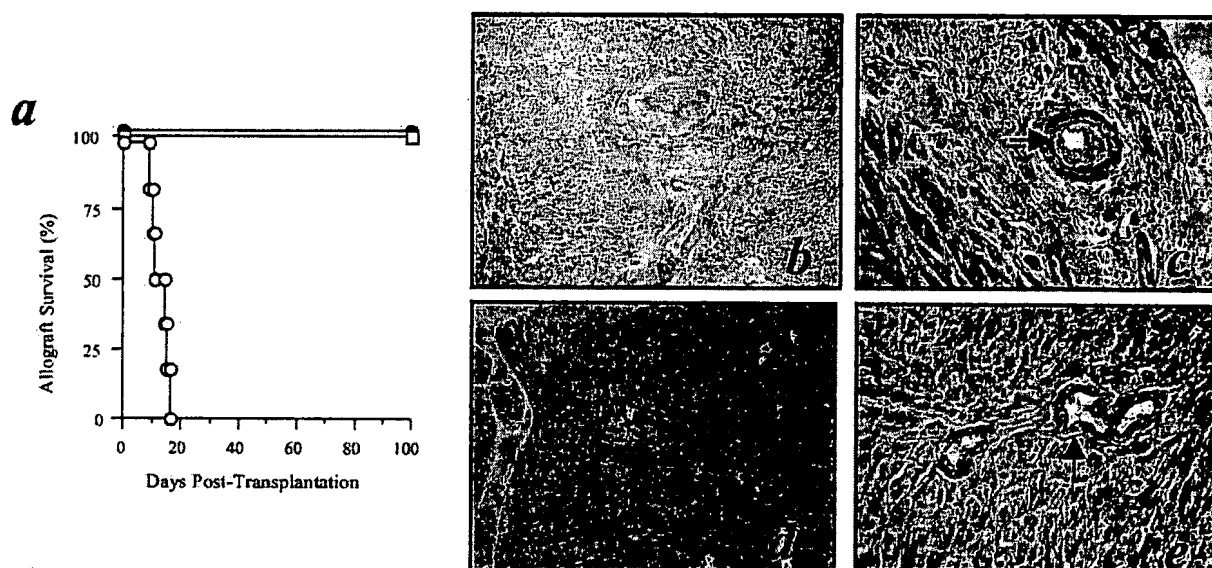
#### *Acute rejection is recapitulated by subjecting a healed-in allograft to ischemia-reperfusion injury*

Healed-in grafts, which have recovered from the sequelae of ischemia-reperfusion, constitute a much less proinflammatory environment than newly transplanted grafts (31), raising the possibility that resolution of ischemia-reperfusion injury is an important mechanism of graft adaptation. We therefore asked whether resubjecting a healed-in allograft to ischemia-reperfusion injury reca-

pitulates the acute rejection process. To answer this question, we retransplanted cardiac allografts that had been parked for 50 days in *aly/aly-spleen* hosts to a second set of *aly/aly-spleen* mice and transferred activated T cells 2 days after the retransplantation procedure. As shown in Fig. 3*a*, all retransplanted hearts underwent clinical rejection (all allografts stopped contracting by day 62 after T cell transfer; median survival time, 44 days), albeit at a delayed time point compared with the newly transplanted hearts (all allografts stopped contracting by day 23 after T cell transfer; median survival time, 20 days,  $p < 0.05$ ). Histologic examination revealed that retransplanted hearts harvested from recipients that received activated T cells failed due to acute cellular rejection (Fig. 3*b*), but that these grafts also developed elements of chronic rejection (vascular intimal thickening) (Fig. 3*c*). Healed-in hearts retransplanted into *aly/aly-spleen* mice that did not subsequently receive activated T cells continued to contract for the full duration of the experiment (Fig. 3*a*). Therefore, resolution of ischemia-reperfusion injury is an important mechanism of graft adaptation that protects against acute rejection.

#### *Activated T cells fail to accumulate in healed-in allografts*

Allograft rejection is mediated by activated T cells that home to and accumulate in the transplanted organ. Therefore, we hypothesized that a healed-in allograft is protected from acute rejection



**FIGURE 2.** Healed-in allografts undergo chronic rejection in immunosuppressed wild-type hosts following the transfer of activated T cells. *a*, Cardiac allografts are acutely rejected in immunosuppressed wild-type hosts if activated T cells are transferred 2 days after transplantation (○,  $n = 6$ ). In contrast, acute rejection is not observed if activated T cells are transferred 50 days after transplantation (□,  $n = 5$ ). Control mice that did not receive any exogenous T cells did not reject their grafts (●,  $n = 5$ ). *b* and *c*, MT (×200) and Verhoess Van Giesen (×400) staining of cardiac allograft tissue removed 50 days after T cell transfer in immunosuppressed mice that received activated T cells 50 days following transplantation. Note diffuse fibrosis (*b*) and vascular intimal thickening (*c*, arrow), which are consistent with chronic rejection. *d* and *e*, MT (×100) and Verhoess Van Giesen (×400) staining of cardiac allograft tissue removed 100 days after transplantation from immunosuppressed mice that did not receive exogenous T cells. Note lack of significant fibrosis (*d*) and normal vessel wall morphology (*e*, arrow).

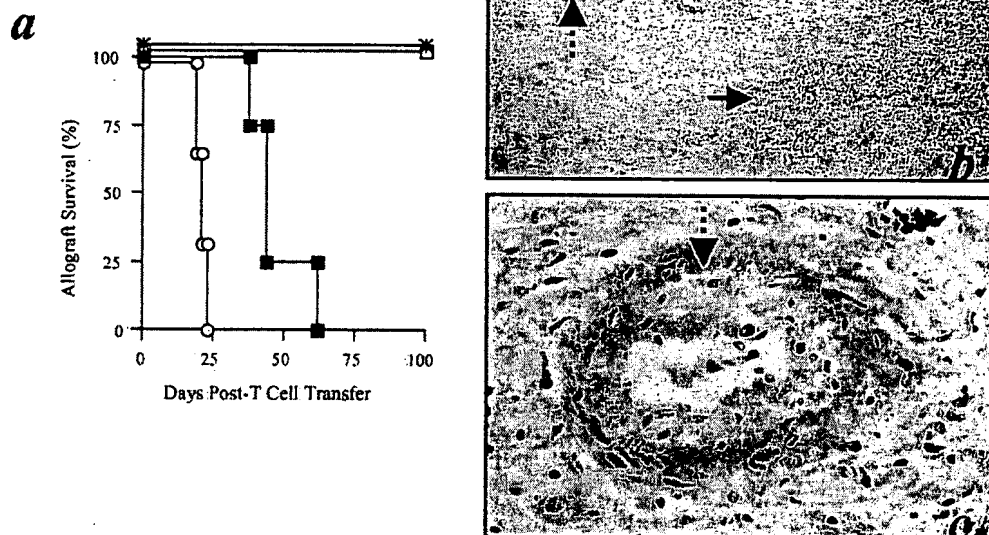
because of reduced homing or accumulation of activated T cells in the graft. To test this hypothesis, we transferred activated T cells, harvested from CD8 TCR-transgenic  $2C^{+/-}$  (H-2<sup>b</sup>) mice 4 days after immunization with BALB/c (H-2<sup>d</sup>) splenocytes, to *aly/aly-spleen* recipients of BALB/c cardiac allografts either 2 or 50 days following transplantation. Approximately 30–50% of CD8 T cells in  $2C^{+/-}$  mice express the transgenic TCR specific to the MHC class I Ag L<sup>d</sup> present on BALB/c cells and are detected with the clonotypic Ab 1B2. Cardiac grafts were harvested either 6 or 24 h after T cell transfer, and infiltrating T cells were phenotyped and quantitated. As shown in Fig. 4*a*, the total number of infiltrating T cells 6 h post-cell transfer was comparable in newly transplanted (day 2) and healed-in cardiac (day 50) allografts. However, at 24 h, the T cell number had increased by ~10-fold in newly transplanted hearts, but declined by 3- to 4-fold in healed-in hearts. This observation was true for both CD4 and CD8 T cells and for CD8<sup>+</sup> 1B2<sup>+</sup> (Ag-specific) T cells that infiltrated the grafts (data not shown). The vast majority of infiltrating T cells had an activated (CD44<sup>high</sup>) phenotype, and the higher number of activated T cells present in newly transplanted hearts could not be attributed to increased proliferation, as CFSE dilution profiles were comparable in day 2 and 50 grafts (histograms not shown). We then asked whether resubjecting healed-in allografts to ischemia-reperfusion injury recapitulates the accumulation of activated T cells observed in newly transplanted hearts. To do so, we retransplanted BALB/c cardiac allografts that had been parked in *aly/aly-spleen* mice for 50 days into new *aly/aly-spleen* recipients and transferred activated T cells 2 days later. As shown in Fig. 4*a*, activated T cells accumulated in retransplanted hearts to the same extent that they would have if the hearts had been newly transplanted. These findings indicate that ischemia-reperfusion injury is a critical determinant of activated T cell accumulation in transplanted organs.

Because T cell entry into nonlymphoid tissues can occur in the context of a nonspecific, Ag-independent response to ischemia-

reperfusion injury, we quantitated the accumulation of activated exogenous T cells in syngeneic cardiac grafts and of endogenous (*aly*) T cells in both syngeneic and allogeneic grafts. We found that activated T cells home to newly transplanted syngeneic grafts at 6 h, but do not accumulate further at 24 h (Fig. 4*b*). A similar population of activated T cells did not infiltrate healed-in syngeneic grafts (<500 cells/graft at either 6 or 24 h) (Fig. 4*b*). These data suggest that the accumulation of activated T cells in nonlymphoid tissues is dependent on the presence of both inflammation and foreign Ags. The accumulation of naive, endogenous (*aly*) T cells in newly transplanted heart allografts was transient, independent of foreign Ag (equal numbers of T cells were isolated from allogeneic and syngeneic grafts), and smaller in magnitude than the accumulation of activated exogenous T cells observed in allogeneic grafts (Fig. 4*c*).

#### Chemokine and adhesion molecule gene expression in newly transplanted and healed-in allografts

To begin to address the mechanisms responsible for the difference in effector T cell accumulation between newly transplanted and healed-in allografts, we compared the expression of key molecules involved in effector T cell homing (chemoattraction) and retention (arrest) in nonlymphoid tissues between day 2 and day 50 cardiac allografts placed in *aly/aly-spleen* recipients. Grafts were harvested before the transfer of activated T cells, and mRNA expression was analyzed by real-time quantitative PCR and by hybridization to murine oligonucleotide microarrays. As shown in Fig. 5, real-time PCR analysis demonstrated that the mRNA levels of four chemokines involved in activated T cell chemoattraction to transplanted organs (IFN-inducible protein-10, monokine induced by IFN-γ (Mig), IFN-inducible T-cell α chemoattractant, and lymphotactin) (3, 32) were significantly elevated in both healed-in and newly transplanted allografts compared with native donor hearts. Although IFN-inducible protein-10 and IFN-inducible T-cell α



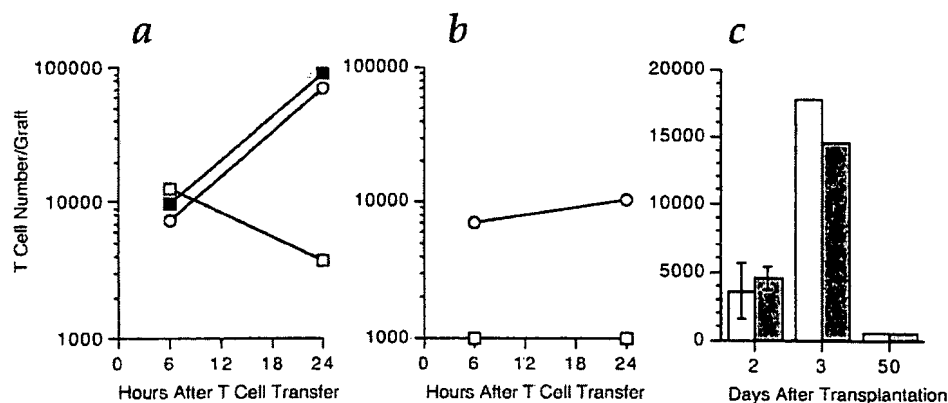
**FIGURE 3.** Ischemia-Reperfusion injury of healed-in allografts recapitulates acute rejection. *a*, Cardiac allografts that had been parked for 50 days in splenectomized *aly/aly* hosts, retransplanted to a second set of splenectomized *aly/aly* mice, and exposed to exogenous activated T cells 2 days after the retransplantation procedure underwent acute rejection (■,  $n = 4$ ). In contrast retransplanted cardiac allografts that were not exposed to exogenous activated T cells did not undergo acute rejection (\*,  $n = 3$ ). The fate of primary cardiac allografts in splenectomized *aly/aly* hosts following the transfer of activated T cells either 2 days (○,  $n = 6$ ) or 70 days (□,  $n = 6$ ) after transplantation is shown for comparison (same groups are also depicted in Fig. 1*a*). *b* and *c*, H&E staining of retransplanted cardiac allograft tissue exposed to exogenous activated T cells and removed at the time of cessation of contractions shows combined elements of both acute and chronic rejection. The straight arrow (*b*) points to the diffuse mononuclear infiltrate characteristic of acute cellular rejection, while the dashed arrows (*b* and *c*) point to a vessel with thickened intima suggestive of chronic allograft vasculopathy.

chemoattractant mRNA levels were lower in day 50 than day 2 allografts, those of Mig and lymphotactin were higher in day 50 grafts. These data indicate that the expression of T cell chemoattractants persists in the transplanted organ even after sufficient time is allowed for the graft to heal. The data are also consistent with our finding that T cells home in equal numbers to day 2 and day 50 allografts early (at 6 h) after adoptive transfer (Fig. 4*a*).

We then asked whether newly transplanted and healed-in allografts differ in the expression of molecules required for arresting activated T cells that have homed to the graft. To address this question, we performed oligonucleotide microarray analysis on cardiac allografts removed either 2 or 50 days after transplantation and before adoptive T cell transfer. Differential gene expression analysis revealed that mRNA species corresponding to 13 extracellular matrix/cell adhesion proteins were significantly less abundant in day 50 than day 2 allografts (Table I). Many of these proteins, particularly laminin and collagens types I and IV, bind to integrins on activated T cells and cause their arrest within inflamed tissues (33, 34). No extracellular matrix protein mRNA species was up-regulated in day 50 relative to day 2 grafts, and no difference in chemokine mRNA expression was detected between the two time points, except for Mig mRNA, which was 3-fold elevated in day 50 grafts. Likewise, there were no significant differences between day 2 and day 50 allografts in the expression of integrin receptor ligands required for the firm adhesion of T cells to the endothelium. Taken together, our gene expression data suggest that diminished accumulation of T cells in healed-in allografts could be attributed at least in part to reduced arrest of effector T cells within the graft.

## Discussion

We have provided direct evidence that the status of the graft plays a dominant role in both allograft survival and the type of rejection that ensues. When exposed to identical populations of activated T cells, newly transplanted heart allografts were rejected acutely, while healed-in grafts survived long-term, but developed histologic manifestations of chronic rejection. This finding was confirmed in two independent adoptive transfer models in which the status of the graft was varied while keeping the alloimmune response constant. In the first model, vascularized cardiac allografts were parked in mice that lack secondary lymphoid organs (*aly/aly-spleen*) for either 2 days (newly transplanted) or >50 days (healed-in) before adoptively transferring wild-type, allosensitized T cells. In the second model, allografts were parked in wild-type mice in which primary immunity was inhibited by agents that block T cell costimulation. Allograft rejection in both models is mediated exclusively by the adoptively transferred, Ag-experienced T cells, and not by endogenous lymphocytes (20, 24, 29, 30). The principal advantage of these models is that they allow one to investigate how graft adaptation, defined as resistance of the graft to the effector arm of the immune response, shapes the outcome of rejection independent of alterations in afferent immunity that occur after transplantation. The finding that healed-in allografts underwent chronic instead of acute rejection in both *aly/aly-spleen* and wild-type hosts makes it unlikely that our results are biased by the immunologic abnormalities present in *aly* mice (26, 27). Moreover, the wild-type model simulates the usual clinical situation whereby transplant recipients are immunosuppressed to

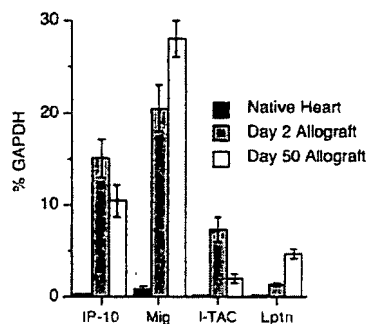


**FIGURE 4.** Intragraft T cell accumulation. *a*, Total graft-infiltrating T cells ( $CD4^{+} + CD8^{+}$  cells) were quantitated at 6 and 24 h after the transfer of activated T cells either 2 days ( $\circ$ ) or 50 days ( $\square$ ) following cardiac allograft transplantation. Retransplantation of an allograft parked for 50 days, followed by the transfer of activated T cells 2 days later, recapitulated intragraft T cell accumulation ( $\blacksquare$ ). *b*, Total intragraft T cell accumulation following the transfer of activated T cells either 2 days ( $\circ$ ) or 50 days ( $\square$ ) after syngeneic cardiac transplantation. *c*, Accumulation of endogenous T cells in the graft following either allogeneic ( $\square$ ) or syngeneic ( $\blacksquare$ ) cardiac transplantation (no exogenous T cells were transferred in these experiments). In all experiments shown, T cells were pooled from three grafts per group. In the day 2 posttransplantation group, the experiment was repeated twice and SD is shown.

prevent acute rejection, yet develop chronic rejection later on. Therefore, our data provide direct evidence that adaptive changes that occur in the graft itself play a dominant role in defining the type of rejection that occurs.

The adaptive mechanisms that account for graft resistance to immune attack are not completely understood. Proposed mechanisms include gradual replacement of graft endothelium by host endothelial cells, reduced expression of MHC Ags in the graft, and resistance of graft cells to apoptosis (12, 35). In this study, we addressed the general hypothesis that ischemia-reperfusion injury that occurs at the time of transplantation favors acute rejection and, conversely, the resolution of ischemia-reperfusion injury over time sways rejection toward a chronic form. To test this hypothesis, we resubjected healed-in allografts to ischemia-reperfusion injury by retransplanting them into new hosts before transferring allosensitized T cells. We found that retransplantation recapitulates acute rejection, albeit partially, indicating that resolution of ischemia-reperfusion injury is an important, but not the only mechanism of graft adaptation. Other changes that occur in long-term surviving grafts, such as the replacement of donor APCs with host APCs and up-regulation of antiapoptotic genes, may protect transplanted tissues against immunologic attack.

Ischemia-Reperfusion injury is a complex inflammatory process that encompasses up-regulation of adhesion molecules, induction of inflammatory mediators, and activation of the complement system (36). Because these events participate in leukocyte migration into peripheral tissues, we asked in this study whether ischemia-reperfusion injury influences the type of rejection by modulating activated T cell entry into the transplanted organ. We found that the status of the allograft (newly transplanted vs healed-in) is a critical determinant of T cell homing to the graft. Interestingly, the number of T cells that entered the allograft at an early time point (6 h after lymphocyte transfer) appeared to be independent of the graft status, while T cell accumulation observed 18 h later occurred only if the graft had been subjected to ischemia-reperfusion injury. This finding suggests that the accumulation, rather than initial homing, of activated T cells is dependent on the presence of inflammation within the target tissue. Moreover, T cell accumulation was also dependent on the presence of foreign Ags, as activated T cells failed to accumulate in newly transplanted syngeneic grafts. These findings are relevant not only to transplantation, but also to the migration of Ag-experienced T cells to sites of infection and autoimmunity. The adoptive transfer models described in this work, therefore, are well suited for analyzing in more depth the



**FIGURE 5.** Chemokine gene expression by real-time quantitative PCR. Cardiac allografts were analyzed either 2 or 50 days after transplantation to *aly/aly*-spleen mice and before adoptive T cell transfer ( $n = 3$ /group). Gene expression in the allografts (mean  $\pm$  SD) is shown in comparison with control, native donor hearts ( $n = 3$ ). mRNA levels of all four chemokines studied were significantly greater in the allografts than in the native hearts. Lptn = lymphotactin.

**Table 1.** Extracellular matrix protein mRNA species down-regulated in healed-in allografts

Gene Accession No.	Description	Fold Reduction (day 50 vs day 2) <sup>a</sup>
L02918	Procollagen type V, $\alpha 2$	4.8
X56304	Tenascin C	4
AF011450	Procollagen type XV	3.6
AF064749	Collagen type V1, $\alpha 3$	3.4
U03419	Procollagen type 1, $\alpha 1$	3.4
X58251	Procollagen type 1, $\alpha 2$	3.1
M15832	Procollagen type IV, $\alpha 1$	2.9
U69176	Laminin $\alpha 4$	2.7
X04647	Procollagen type IV, $\alpha 2$	2.6
Z18272	Procollagen type V1, $\alpha 2$	2.4
U12147	Laminin $\alpha 2$	2.3
AB009993	Collagen type V, $\alpha 1$	2.1
X05212	Laminin B1	2.0

<sup>a</sup> Three samples were analyzed per group. Values shown are average fold reduction determined by differential gene expression analysis of microarray data.



factors that govern the accumulation of effector and memory T cells in nonlymphoid tissues.

Diminished T cell accumulation in healed-in allografts could be due to either decreased arrest and survival of T cells or a reduction in T cell infiltration after the initial homing stage. Chemokine and adhesion molecule gene expression analysis reported in this study does not definitively differentiate between these possibilities, but suggests that diminished T cell accumulation in healed-in allografts is caused by reduced arrest of activated T cells due to decreased expression of extracellular cell matrix proteins involved in cell adhesion. Additional analysis is needed to exclude the possibility that following adoptive T cell transfer, infiltrating cells increase chemokine expression and lead to further T cell accumulation in newly transplanted, but not healed-in allografts. Moreover, it is possible that the final outcome of rejection (acute vs chronic) is not simply determined by the number of activated T cells that accumulate in the graft, but also by the locale of T cell accumulation (37). For example, it is conceivable that vascular T cell accumulation leads to chronic rejection, while parenchymal T cell localization leads to acute rejection. This hypothesis remains to be tested.

Bingaman et al. (31) observed that healed-in skin or vascularized cardiac allografts parked for 50 days in *Rag*<sup>-/-</sup> lymphocyte-deficient recipients undergo acute rejection upon the transfer of exogenous T cells. In contrast, we found in this study that healed-in vascularized allografts undergo chronic rejection. The discrepancy between our results and theirs could be attributed to a fundamental difference in the models used. In the model used by Bingaman et al., the homeostatic proliferation of T cells transferred to lymphocyte-deficient *Rag*<sup>-/-</sup> mice could make these cells more aggressive, leading to acute instead of chronic rejection (38). In contrast, T cells transferred to *aly/aly-spleen* mice do not undergo significant homeostatic proliferation, as these mice are T cell repopulated (39).

Our study differs from previous investigations into the role of the graft in the rejection process in that we used experimental models in which the afferent and efferent limbs of the immune response are separated. Earlier studies by Lechler and Batchelor and by Rosengard and colleagues (10, 11) clearly demonstrated the importance of the afferent arm of the immune response, specifically that of passenger APCs, in determining the survival of a transplanted organ and the type of rejection that ensues. In this study, we focused on how the graft modulates the efferent limb of the immune response and found, contrary to prevailing view, that the graft itself plays a dominant role in defining the type of rejection even after a full-blown alloimmune response has been initiated. Our finding may explain why many patients who stop their immunosuppression several years after transplantation develop chronic instead of acute rejection, and may provide insights into harnessing graft adaptation to achieve long-term allograft acceptance.

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Acute rejection remains an important clinical challenge, and accurate diagnosis is contingent on the invasive procedure of renal allograft biopsy. Core needle biopsies, while providing invaluable information, carry with them the risk of significant hematuria, arteriovenous fistulas, or graft loss. Successful development of a noninvasive surrogate for allograft biopsies, therefore, has the significant potential for improving transplant patient management. We have initiated studies investigating the correlation between histologic diagnosis of acute rejection and urinary cell cytotoxic attack molecule mRNA steady-state levels. RT-quantitative polymerase chain reaction was used to quantify mRNA encoding perforin or granzyme B in urinary cells. Renal allograft biopsies were classified on the basis of Banff criteria, and the mRNA steady-state levels were correlated with the histologic diagnosis. Our data suggest that the level of perforin transcripts or that of granzyme B transcripts in urinary cells is significantly higher during histologically validated acute rejection compared with no rejection.

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### CYTOKINE GENE POLYMORPHISMS

*Ian V. Hutchinson, Ph.D.*

The production of cytokines is under genetic control. Alleles of the cytokine genes encode high or low production, and because these segregate independently, each one is a mosaic of higher or lower responses. In the context of transplantation, the cytokine genes we have studied are those for the inflammatory cytokines tumor necrosis factor-alpha (TNF-alpha) and interferon-gamma (IFN-gamma) and the anti-inflammatory/immunoregulatory cytokines interleukin-10 (IL-10) and transforming growth factor-beta 1 (TGF-beta 1). TGF-beta 1 also has potent fibrogenic activities. We have used simple polymerase chain reaction-based methods to genotype kidney, heart, lung, and liver transplant recipients for these cytokines. Acute cellular rejection is strongly associated with high-producer TNF-alpha genotype in heart and kidney recipients, whereas IL-10 and IFN-gamma play modulating roles. Chronic rejection (including declining graft function, transplant vasculopathy, graft loss, and patient death) is strongly associated with high-producer TGF-beta 1 genotype. These results imply that TNF-alpha and TGF-beta 1 are pivotal cytokines in the acute and chronic transplant rejection.

**EXHIBIT**

E

## Key References

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Saint Louis, Missouri 63110, USA.

Current strategies for immunosuppression following organ transplantation focus on the prevention of acute rejection. As new generations of immunosuppressants have been developed, acute rejection rates have diminished markedly. The new challenge, then, is to prevent the devastating complications of chronic rejection, which have remained largely unchanged over the decades. The process of chronic rejection is a complex one, and it is likely that most, if not all, components of the immune system play some role in the long-term, smoldering failure of organs following transplantation. Through a better understanding of their individual contributions as well as interactions, new strategies may be developed to overcome this problem. We present here an overview of the major immune components thought to be involved in chronic rejection.

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